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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Staphylococcus aureus small colony variants: Prevalence in chronic rhinosinusitis and induction by antibiotics

To the Editor,

Staphylococcus aureus (*S. aureus*) small colony variants (SCVs) are an altered bacterial phenotype characterized by slow growth rate and small colony size. Due to reduced antimicrobial sensitivity and a capacity to invade host cells, *S. aureus* SCVs can escape immune defense mechanisms and contribute to therapy-refractory Staphylococcal infections (reviewed in Ref. 1). *Staphylococcus aureus* SCVs have been isolated from sites of several persistent and relapsing chronic infections such as osteomyelitis² and from within the nasal polyps of patients with chronic rhinosinusitis (CRS)³; however, the triggers and mechanisms of SCV formation are largely unknown. Previous work has demonstrated *S. aureus* can be induced into the SCV phenotype by exposure to different stressors in vitro^{2,4,5} and SCVs are also isolated from patients with ongoing antibiotic treatment.^{1,6-8} However, the incidence and characteristics of SCVs amongst *S. aureus* clinical isolates (CIs) from CRS patients and whether these isolates are induced into a SCV phenotype by clinically relevant antibiotics are not known. As antibiotics form the mainstay of treatment for CRS patients, this information is important to advise treatment options.

The purpose of this study was to determine the prevalence of *S. aureus* SCVs in CRS CIs, analyze the capacity of clinically relevant antibiotics to induce SCVs, and compare the characteristics of induced SCVs with their normal colony phenotype (NCP) parents. To achieve this, the SCV status of 66 CRS *S. aureus* CIs was determined and 11 types of antibiotics tested for their capacity to induce SCVs in representative CIs and the reference strain ATCC51650.

SCVs were defined by decreased colony size and reduced pigmentation compared to parent NCP. Biochemical activity as well as infection ability, antibiotic sensitivity, and stability was also tested. Details of materials and methods are found in this article's online repository. Of the 66 *S. aureus* CIs from CRS patients assessed for SCV phenotype, half (33/66) grew large-sized white or yellow colonies, typical of *Staphylococci* and 26 of 66 *S. aureus* CIs grew small (≤ 1 mm) colonies with some of these (5/26) appearing white in color and lacking hemolytic activity (SCVs). Mixed populations of small and large colonies were present in the remaining 7 of 66 CIs with three of these containing colonies with SCV phenotype (Table S1). Representative images of *S. aureus* CIs are shown in Figure S1.

After SCV status was determined, we then tested 11 antibiotics for their capacity to induce SCVs of ATCC51650 and three representative CIs. The MIC of these antibiotics was determined and is shown in Table S2. Incubation with gentamicin was particularly effective at inducing SCVs. This was consistent with previous reports that have shown gentamicin exposure induces reversible rapid switching of gentamicin-sensitive strains into gentamicin-resistant SCVs.⁵ Isolates CI1 and ATCC51650 showed switching to SCV phenotype after as little as 1 hour at $\frac{1}{2}$ MIC whilst all isolates showed a gradual increase in SCVs over the 7-day period in continuous culture with two MIC gentamicin (Figure 1A and Figures S2 and S3). The other antibiotics tested did not induce SCVs at short exposure times (<24 hours). However, SCVs were induced by 24-hour incubation with clindamycin from three MIC to 10 MIC and by rifampicin from eight MIC to 10 MIC (Figure 1B-D). Using different protocols, Tuchscher et al² similarly showed gentamicin- and clindamycin-induced *S. aureus* SCV formation and reported a high

resistance rate of bacteria when exposed to rifampicin for 24 hours. Our results add to these findings, demonstrating that bacteria exposed to high concentrations of rifampicin were rifampicin-resistant and of a SCV phenotype. Stability testing of the isolates indicated SCVs induced by short exposure times to gentamicin (<2 hours) reverted back to NCP when cultured overnight. However, incubation with two MIC gentamicin for 3 days or more induced SCVs that were stable for up to 5 days incubation. The phenotypic instability of SCVs is a known phenomenon, with phenotype reversion occurring frequently after incubation in stress-free conditions making laboratory-based investigation of clinically isolated SCVs difficult.¹

The catalase, coagulase, and hemolytic activity as well as infection ability and antibiotic sensitivity of one representative SCV

clinical isolate (CI1-SCV) was also assessed. CI1-SCVs were induced by gentamicin, clindamycin, and rifampicin for 4 days and were stable for at least 5 days continuous culture in antibiotic-free broth. All antibiotic-induced CI1-SCVs lost their coagulase activity and kept catalase activity, with loss of haemolytic activity only observed in gentamicin- and clindamycin-induced SCVs. Induced SCVs were also resistant to the antibiotics used for induction but more sensitive to other antibiotics (Table 1). CI1-SCVs also showed lower infection rate compared to the NCP parent strain (32.7% vs 76.1% for the CI1-SCVs and CI1, respectively) when applied to human bronchial epithelial (HBE) cells for 6 hours. However, the percentage of infected cells decreased rapidly for the CI1-NCP after extracellular bacteria were removed, and after 24 hours, the CI1-SCVs infection rate was similar to the CI1-NCP parents (18.3% vs 22.2%; Figure S4).

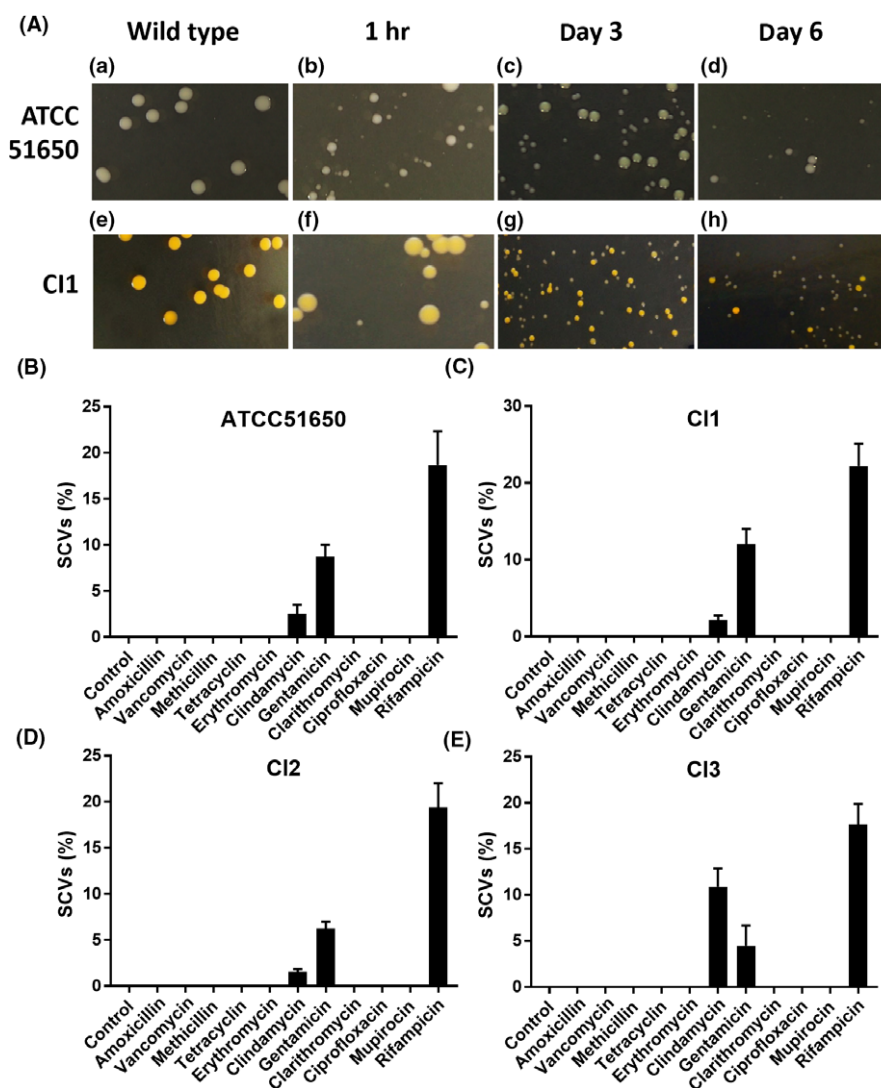


FIGURE 1 Induction of SCVs by antibiotics after 24-h culture in TSB: (A) representative plate images of gentamicin-induced ATCC51650 and CI1 *Staphylococcus aureus* SCVs. ATCC 51650 and CI1 parental strains were observed as large colonies (a, e); continuous incubation with two MIC gentamicin induced an increasing number of SCVs over time, shown at 1 h (b, f), day 3 (c, g), and day 6 (d, h) as small colonies with reduced pigmentation; (B-E) induction of SCVs in representative isolates ATCC51650 (B), CI1 (C), CI2 (D), and CI3 (E) by 11 different clinically used antibiotics. Clindamycin, gentamicin, and rifampicin were the only antibiotics observed to induce SCV phenotype [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Characteristics of CI1-SCVs induced by gentamicin, clindamycin and rifampicin for 4 d

	G-SCVs	C-SCVs	R-SCVs	NCP-parents
Catalase	Positive	Positive	Positive	Positive
Coagulase	Negative	Negative	Negative	Positive
Hemolysis	Negative	Negative	Positive	Positive
MIC-Gent	8~16 ^a	1	1	2
MIC-Clin	0.04 ^b	>10 ^a	0.04 ^b	0.2
MIC-Rif	<0.001	<0.001	0.625~2.5 ^a	0.001~0.004
MIC-Met	0.39	0.78	0.39	0.39~0.78
MIC-Ery	0.39 ^b	0.39 ^b	0.2~0.39 ^b	0.78

(MIC-Gent), (MIC-Clin), (MIC-Rif), (MIC-Met), (MIC-Ery): MIC ($\mu\text{g/mL}$) to gentamicin, clindamycin, rifampicin, methicillin and erythromycin respectively; C-SCVs: SCVs induced by clindamycin; G-SCVs: SCVs induced by gentamicin; R-SCVs: SCVs induced by rifampicin.

^aMIC of SCVs significantly higher than parents ($P < 0.05$).

^bMIC of SCVs significantly lower than parents ($P < 0.05$).

Both phenotypes could survive in the cells for more than 4 days. This suggests that the CI1-SCVs, whilst not as capable of initiating intracellular infection, are better able to persist in the intracellular environment. Previous research has indeed shown intracellular infection with *S. aureus* SCVs induced a less widespread upregulation of pro-inflammatory factors in comparison with intracellular infection with NCP parent strains⁹ which may in part explain these results.

In conclusion, 12% of *S. aureus* CIs from the sinonasal cavities of CRS patients had colonies with characteristics reminiscent of SCVs when cultured *in vitro*. Moreover, *S. aureus* CIs could be induced into antibiotic-tolerant SCVs by prolonged exposure to different clinically used antibiotics. In our experiments, antibiotic-induced *S. aureus* SCVs were tolerant to the antibiotics used for induction, but were more sensitive to alternative antibiotics tested. These findings may have important implications for the design of optimal antibiotic treatment strategies for relapsing infections in the context of CRS.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Online repository- MATERIALS AND METHODS

Clinical isolates, cells and antibiotics

The study was approved by The Queen Elizabeth Hospital (TQEH) Human Research Ethics Committee and written informed consent was obtained from all study participants. *S. aureus* ATCC51650 was obtained from the American Type Culture Collection (Manassas, VA, USA). 66 clinical *S. aureus* isolates (CIs) were isolated from the sinonasal cavities of CRS patients (CRSwNP and CRSsNP) at TQEH (Woodville, SA, Australia). Patients did not receive antibiotic therapy at the time of collection and exclusion criteria included antibiotic usage in the 4-week period prior to study initiation. In each case, *S. aureus* was cultured by an independent institute and antibiotic susceptibility was determined by disc diffusion on Mueller-Hinton agar per Clinical and Laboratory Standards Institute (CLSI) recommendations (Adelaide Pathology Partners, Mile End, SA, Australia). Bacteria were isolated on Columbian horse blood agar plates, colistin nalidixic acid plates, or cystine lactose electrolyte-deficient plates (Oxoid, Thebarton, SA, Australia). Positive cultures were confirmed to be *S. aureus* using latex agglutination testing. 11 kinds of antibiotics were tested, including amoxicillin, vancomycin, methicillin, tetracycline, erythromycin, clindamycin, gentamicin, clarithromycin, ciprofloxacin, mupirocin and rifampicin (Sigma, USA).

Determination of the Minimum Inhibitory Concentration (MIC) of antibiotics

The Minimum Inhibitory Concentration (MIC) of antibiotics was assayed using the broth microdilution technique for MIC determination as described previously (1). In brief, 5×10^5 CFU/ml of test bacteria were inoculated into each well of a 96-well plate with tryptone soya broth (TSB). Serial dilutions of antibiotics were subsequently added. The MIC value was recorded as the lowest concentration at which there was no visible bacterial growth. 10 μ l samples were plated on tryptone soya agar (TSA) to determine CFU/ml.

S. aureus* SCVs induction by antibiotics *in vitro

To explore if antibiotics can induce clinical *S. aureus* isolates into SCVs, three representative clinical isolates with standard colonies, designated CI 1-3, were randomly selected. ATCC51650 was also included. In brief, 5×10^5 CFU/ml bacteria were grown in 10 ml TSB with antibiotics at MIC concentration for 24 h at 37°C in 5% CO₂ with shaking at 180rpm. The culture was subsequently centrifuged (3000 rpm, room temperature for 5 minutes) prior to aspiration of the supernatant and repetition of the cycle for 7 days. Every 24 hours, a sample of the broth was plated onto TSA plates to observe for colony phenotypes. SCVs were defined as white color and small size (<1/10 size of parental colonies) when grown on agar plates as described previously (2). Colonies on TSA plates were measured at fixed time points and classified into Normal Colony Phenotype (NCP) or SCVs. The number of SCVs as a percentage of the total population was determined by serial plating and colony counts over time. Bacteria were also tested in a similar manner at 2x, 3x, 4x, 6x, 8x and 10x MIC concentrations of antibiotics.

Catalase, coagulase and hemolytic activity

Catalase and coagulase activity of bacterial strains was determined by suspending cells in 0.9% saline in a glass tube. Hydrogen peroxide was added followed by observation of gas formation, indicating catalase activity. The coagulase activity was determined by observation of clumping of bacterial cells after addition of human plasma. Hemolytic

activity of putative SCVs was determined by streaking out bacteria on Oxoid sheep blood agar (Oxoid, Australia), followed by incubation at 37°C for 24 h and observation of a hemolysis zone.

***S. aureus* SCV stability assessment**

Antibiotics induced SCV stability was tested by sub-culturing individual small colonies onto TSA plates overnight under antibiotic-free conditions (37°C, 24 h). A single SCV colony from the sub-culture was then cultured in 10 ml TSB without antibiotics for 24 h, 37°C in 5% CO₂ with shaking at 180rpm. Phenotypes were assessed after culturing samples onto TSA plates for 24 h from TSB liquid medium. This culture cycle was repeated until the parental phenotype was observed.

Cell culture of human bronchial epithelial cells and infection with gentamicin induced *S. aureus* SCVs

Human bronchial epithelial cells (16HBE14o-HBE'014) were a kind gift from Dr Dieter Gruenert and cultured in Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum (FCS), 1% L-Glutamine and 1% penicillin / streptomycin (Gibco, Carlsbad, CA). HBE cells were then grown to 65% confluence in 8-well glass chamber slides (Corning Inc, Mulgrave, Australia) and washed with PBS prior to infection with *S. aureus*. Gentamicin induced CI1-SCVs and their parental phenotype CI1 were used to infect HBE cells. In brief, bacteria were collected after overnight incubation at 37°C on TSA agar plates, re-suspended in MEM to 1.0 McFarland units and incubated with HBE cells for six hours (multiplicity of infection (MOI) of 100:1) at 37°C in 5% CO₂. The cells were then washed three times in PBS, incubated with MEM containing 10 ug/ml lysostaphin for 15 minutes, washed 3 times in PBS and incubated in MEM supplemented with 10% FCS and 1% L-Glutamine at 37°C in 5% CO₂. Media was changed every 24 hours. At specific time points, cells were fixed using methanol for 7 minutes and stained using Giemsa stain for 1 hour. Slides were inspected using a light microscope (Eclipse 90i, Nikon, Japan) at 60X magnification with oil immersion. Minimally two hundred cells were inspected per well to determine the percentage of infected cells.

Statistical analysis

GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA) was used to convert data to a logarithmic scale and to remove variation between replicates. Statistical significance for SCVs MIC results was computed using a one-way analysis of variance (ANOVA). Student's t-test was used to compare SCVs infection rate with parent strains in HBE cells. Significance was determined at a p value <0.05.

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Supplementary figures/ tables

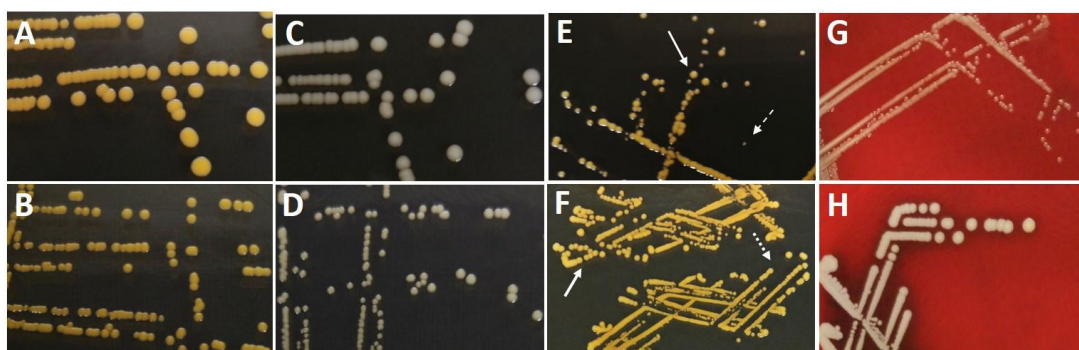
Supplementary Table 1 MIC ($\mu\text{g/ml}$) values of ATCC51650 and clinical isolates (CI1-3)

antibiotics	MIC ($\mu\text{g/ml}$)			
	ATCC	CI1	CI2	CI3
Amoxicillin	0.78	3.12	0.1	0.39
Vancomycin	0.78	0.2	1.56	0.39
Methicillin	0.78	0.78	0.39	0.39
Tetracyclin	0.16	0.16	0.08	0.08
Erythromycin	0.78	>50	0.78	>50
Clindamycin	0.2	0.2	0.2	0.2
Gentamicin	2	1	2	4
Clarithromycin	0.2	>50	0.2	0.39
Ciprofloxacin	0.5	0.5	0.5	0.5
Mupirocin	1	2	1	2
Rifampicin	0.002	0.004	0.001	0.004

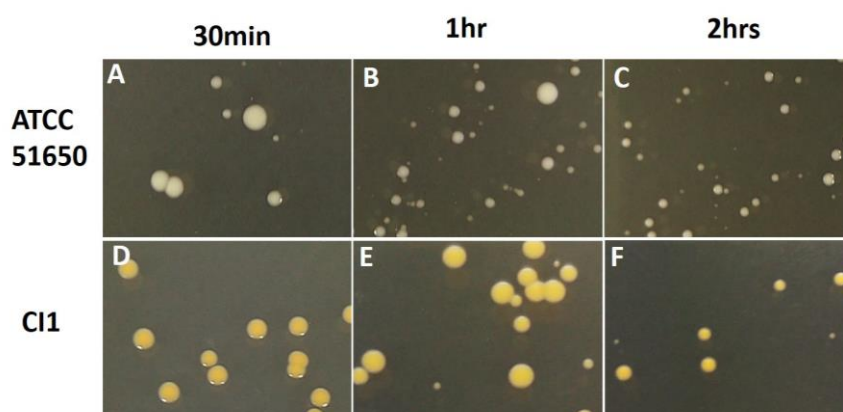
MIC=minimum inhibitory concentration

Supplementary Table 2 Summary color and size of 66 CRS *S. aureus* clinical isolates

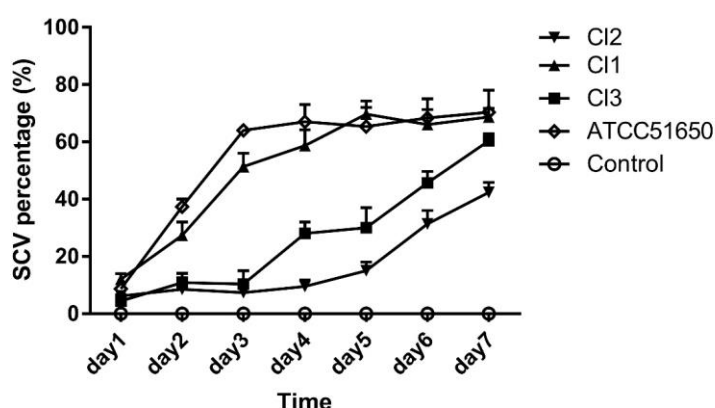
		Size		
		$\leq 1\text{mm}$	$> 1\text{mm}$	Mixed
Color	White	5	6	3
	Yellow	21	27	0
	Mixed	-	-	4
Total		26	33	7



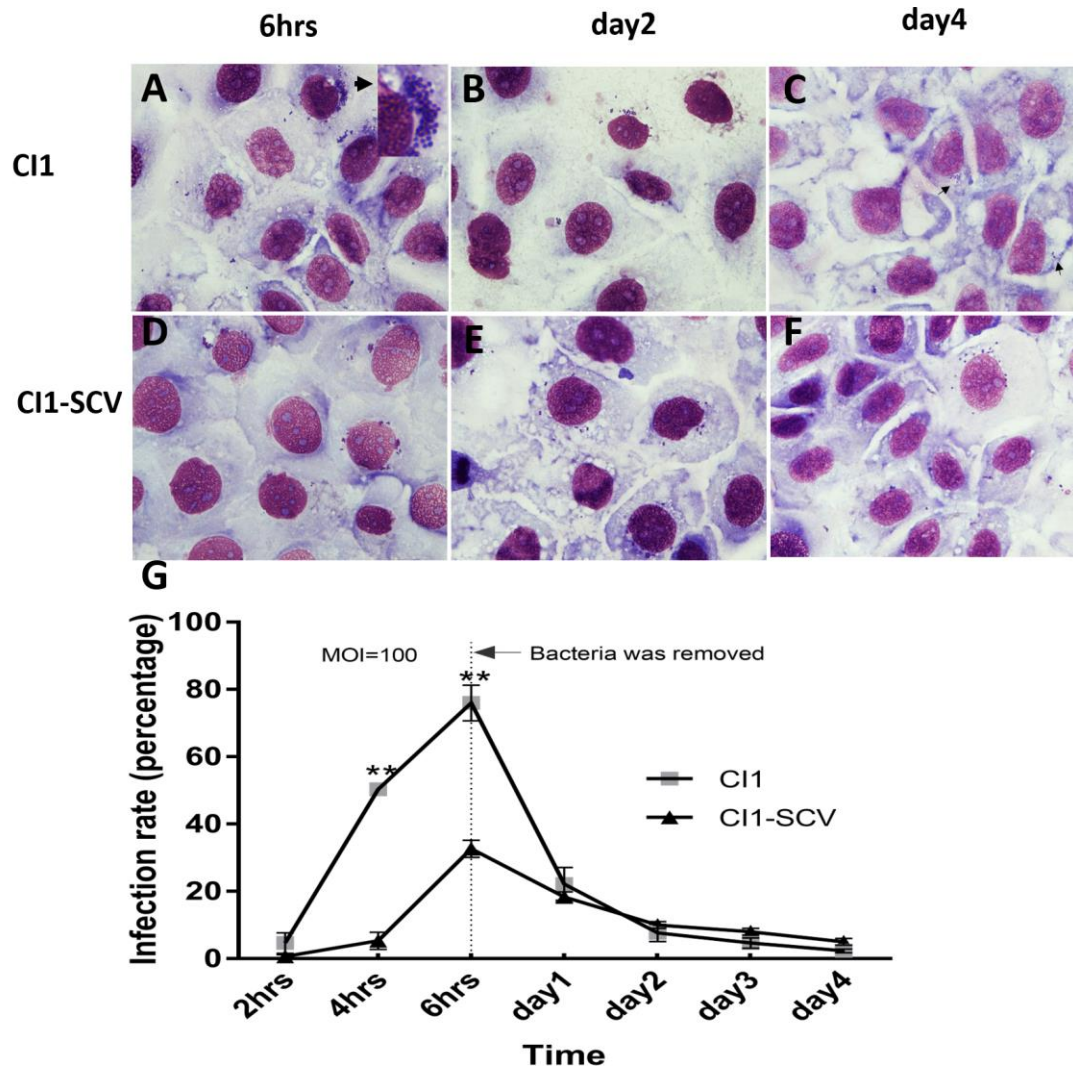
Supplementary Figure 1. Representative images of *S. aureus* clinical isolates. *S. aureus* colonies present as large, yellow (A), small, yellow (B), large, white (C), small, white (D), mixed size with bigger colonies (E solid arrow) and small white colonies (E broken arrow). After overnight sub-culture on TSA plates, the small colony in the mixed population reverted to a larger size (F). One isolate grew SCV colonies without hemolytic activity (G) but reverted to large hemolytic colonies after 6 days culture in TSB medium (H).



Supplementary Figure 2. *S. aureus* SCVs induction under brief gentamicin stress. ATCC 51650-SCVs were observed after 30 minutes 2MIC gentamicin stress (A), with increasing numbers of SCV colonies for ATCC 51650 and C1 after 1 hour (B, E) and 2 hours (C, F) gentamicin incubation.



Supplementary Figure 3. Percentage of SCVs for ATCC51650, CII-3 induced by Gentamicin (2MIC) incubated for 7 days. Control is antibiotic free TSB medium.



Supplementary Figure 4. Percentage of infected Human Bronchial Epithelial (HBE) cells incubated with CI1 and CI1-SCVs over time. T-test was used to compare the infection rate between CI1 and CI1-SCV. ** $P < 0.01$